



# Cell Division Cycle 6, a Mitotic Substrate of Polo-Like Kinase 1, Regulates Chromosomal Segregation Mediated by Cyclin-Dependent Kinase 1 and Separase

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**CLASSIFICATION: BIOLOGICAL SCIENCES –CELL BIOLOGY**

**Cdc6, a Mitotic Substrate and Partner of Plk1, Regulates Chromosomal Segregation**

**Mediated by Cdk1-separase**

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The authors declare no conflict of interest.

**GenBank accession no.** Human Cdc6: NM\_001254; Human Plk1: NM\_005030.

## **ABSTRACT**

Defining the links between cell division and DNA replication is essential for understanding normal cell cycle progression and tumorigenesis. In this report we explore the effect of phosphorylation of Cdc6, a DNA replication initiation factor, by Plk1 on the regulation of chromosomal segregation. In mitosis, the phosphorylation of Cdc6 was highly increased, in correlation with the level of Plk1, and conversely, Cdc6 is hypo-phosphorylated in Plk1-depleted cells, although cyclin A- and cyclin B1-dependent kinases are active. Binding between Cdc6 and Plk1 occurs through the polo-box domain of Plk1, and Cdc6 is phosphorylated by Plk1 on T37. Immunohisto-chemistry studies reveal that Cdc6 and Plk1 colocalize to the central spindle in anaphase. Expression of T37V mutant of Cdc6 (Cdc6-TV) induces binucleated cells and incompletely separated nuclei. Wild type Cdc6 but not Cdc6-TV binds Cdk1. Expression of wild type Plk1 but not kinase-defective mutant promotes the binding of Cdc6 to Cdk1. Cells expressing wild type Cdc6 display lower Cdk1 activity and higher separase activity than cells expressing Cdc6-TV. These results suggest that Plk1-mediated phosphorylation of Cdc6 promotes the interaction of Cdc6 and Cdk1, leading to the attenuation of Cdk1 activity, release of separase, and subsequent anaphase progression.

## INTRODUCTION

Protein modification such as phosphorylation is an important mechanism for regulating transitions through cell cycle phases to ensure DNA duplication and segregation of chromosomes into daughter cells. Polo-like kinase 1 (Plk1) is an essential mammalian mitotic kinase (1-5). Plk1 is expressed in the S, G2, and M phases of the cell cycle, and its activity peaks in mitosis (2-5). During mitosis, Plk1 localizes to several locations and it has diverse substrates and functions (5). Recently, to gain insight into a connection between cell division and DNA replication, other cell cycle functions of Plk1 have been studied. Plk1 interacts and co-localizes with MCMs and Orc2 in the centrosome (6, 7), suggesting that Plk1 may influence components of DNA replication. Plk1 is known to phosphorylate Hbo1, a histone acetyltransferase binding to Orc1 of pre-replication complex (pre-RC)(8) and Topoisomerase II  $\alpha$  (9), suggesting that Plk1-associated phosphorylation of Hbo1 or Topoisomerase II  $\alpha$  is essential for S phase.

DNA replication is tightly coordinated with cell division in order to maintain genomic integrity. In late mitosis and early G1, replication origins are licensed for replication when origin replication complex (Orc), Cdc6, Cdt1, and Mcm2-7 are loaded (10, 11, 12). Origins harboring pre-RC are licensed for replication but do not initiate DNA synthesis until S phase (10, 11, 12). Cdc6 plays a key role in origin licensing (13, 14). The level of Cdc6 fluctuates during the cell cycle and is controlled by a degradation mechanism that is more active in S phase than in mitosis (15). The abundance of Cdc6 through DNA synthesis indicates that Cdc6 may be required after the initiation of DNA replication. In yeast the ectopic expression of Cdc6 interferes with progression through G2 and causes a dramatic delay in entry into mitosis (13, 14). Overexpression of a dominant-negative Cdc6 mutant induces mitotic delay correlated with inhibition of mitotic cyclin-dependent kinase

(CDK) activity (15). The amino-terminal domain of Cdc6 tightly binds to mitotic CDK (16), suggesting that Cdc6 acts as an inhibitor of mitotic CDK in mitosis. Recent reports show that mitotic Cdc6 stabilizes anaphase-promoting complex/cyclosome (APC/C) substrates and affects modulation of APC/C<sup>Cdc20</sup> (17). Cdc6 is also associated with the mitotic apparatus in mice throughout M phase (18). These studies suggest that Cdc6 plays a role in mitotic progression, but the details remain unclear.

In this report, we explore the interaction between Plk1, a mitotic kinase, and Cdc6, a DNA replication initiation factor. We show that Plk1 phosphorylates Cdc6 and that phosphorylation of Cdc6 by Plk1 promotes interaction of Cdc6 and Cdk1, suggesting that phosphorylation of Cdc6 by Plk1 regulates mitotic exit through Cdk1-separase.

## RESULTS

### **The increased phosphorylation of Cdc6, a pre-replication complex component, was correlated with the level of Plk1 in M phase.**

We have found that Plk1 depletion reduces the loading of Mcm proteins on chromatin and DNA synthesis, which suggests that Plk1 may affect DNA replication (19). To investigate the correlation between Plk1 and the DNA pre-RC components, the levels of pre-RC components including Orc2, Mcm7, Cdc6 and Cdt1 were observed during cell cycle progression. Cells were synchronized with a double thymidine block and released with fresh medium. Nine hours after release, most cells were in M phase and the level of Plk1 increased. The levels of Cdc6 also increased, accompanied by the appearance of a more slowly migrating band. Other pre-replication factors such as Orc2, Mcm7, Cdt1, and geminin were not greatly altered 9 h after release (Fig. S1A). To observe whether pre-RC components are phosphorylated in mitosis, cell extracts were treated with alkaline

phosphatase, when cells were in mitosis determined by FACS analysis (Fig. S1A, Fig. 1A). Phosphatase treatment markedly altered the migration of the upper bands of Cdc6 and Plk1, indicating that the shifted bands were phosphorylated (Fig.1A). In contrast, phosphatase had no effect on the levels of Cdt1 and Mcm7. Consistent with phosphorylation in M phase, the more slowly migrating bands of Cdc6 were detected in nocodazole-treated cells but not in hydroxyurea-treated cells (Fig. S1B).

**Cyclin A- or cyclin B1-dependent kinases are activated but Cdc6 is nonphosphorylated in Plk1-depleted cells.**

The results of phosphatase treatment of mitotic cell lysates suggest that Cdc6 may be phosphorylated by Plk1. To investigate whether the depletion of Plk1 affects the phosphorylation of Cdc6, HeLa cells were infected with lentivirus expressing human Plk1-targeting shRNA and selected with puromycin for 2 days. As shown in Fig. 1B, phosphorylation of Cdc6 was significantly reduced in Plk1-depleted cells. Plk1 depletion over 3 days induces mitotic arrest and apoptosis (20). To avoid these effects, puromycin selection was eliminated and cells were harvested one day after infection. Under these conditions, Plk1-depleted cells cycled slowly and apoptotic cells did not appear during the period examined. In control cells, the phosphorylation of Cdc6 was observed from 6 to 12 hours after release when cells passed through G2 and mitosis. However the phosphorylation of Cdc6 was not detectable in Plk1-depleted cells (Fig. 1C), indicating that the presence of Plk1 affects the phosphorylation of Cdc6.

Cdc6 is a substrate of Cdk2 (21, 22, 23) raising the possibility that a reduction of Cdc6 phosphorylation in Plk1-depleted cells results from the low activities of CDKs. Thus, the activities of cyclin A/Cdk2 and cyclin B1/Cdk1 in Plk1-depleted cells were examined.

Cyclin A- or cyclin B1-dependent kinase assays were performed using immunoprecipitation of cyclin A- or cyclin B1-dependent kinase from hydroxyurea or nocodazole treated cells (Fig. 1, D-G). The activities of cyclin A-associated kinase (Fig. 1D) and cyclin B1-associated kinase (Fig. 1F) in Plk1-depleted cells were greater than in control cells. However, Cdc6 was hypo-phosphorylated in Plk1-depleted cells (Fig. 1, E and G), indicating that the phosphorylation level of Cdc6 was not related to the activity of CDKs in Plk1-depleted cells.

#### **Cdc6 binds to the C-terminal domain of Plk1 *in vitro* and *in vivo*.**

The C-terminal polo-box domain of Plk1 functions as a phosphopeptide-binding domain and may contribute to substrate specificity (24, 25). To determine whether Plk1 interacts with Cdc6 or not, *in vitro* GST pull-down assays were performed. Purified GST-fused Cdc6 (GST-Cdc6) was incubated with lysates of HEK 293T cells expressed with Flag-tagged Plk1 (FLAG-Plk1). As shown in Fig. 2A, FLAG-Plk1 associated with GST-Cdc6, but not with GST. To determine whether the Polo-box influences Plk1 association with Cdc6, Flag-tagged wild type (WT), polo-box mutant which is three points mutant at W414F, V415A, and L427A (FAA), N-terminal mutant (amino acids region 1-305), and C-terminal mutant (amino acids region 306-603) of Plk1 were expressed in HEK 293T cells (Fig. 2B and S2A). Purified GST-Cdc6 fusion protein was incubated with cell lysates expressed wild type or mutant of Plk1. Shown in Fig. 2B (left panel), GST-Cdc6 associated with the wild type of Plk1. However, the binding between GST-Cdc6 and Plk1-FAA mutant was markedly reduced compared to the interaction between GST-Cdc6 and Plk1-WT. GST-Cdc6 also bound the C-terminal Polo-box domain, but not the N-terminal kinase domain of Plk1, indicating that the C-terminal Polo-box domain of Plk1 contains the Cdc6 binding

domain. Endogenous Cdc6 bound to endogenous Plk1 and ectopically expressed Plk1-WT as well (Fig. S2B). As with GST-Cdc6, endogenous Cdc6 associated with the C-terminal domain of Plk1, but not the N-terminal domain (Fig. S2B). From these results we conclude that Cdc6 associates with Plk1 through the C-terminal Polo-box domain of Plk1.

### **Cdc6 is a mitotic binding partner and substrate of Plk1.**

Cdc6 works as a DNA replication initiator in early S phase (10-13) and Plk1 is expressed from S phase and peaks in M phase (4). To investigate when Plk1 interacts with Cdc6, co-immunoprecipitation assays were performed using lysates from cells synchronized in S phase or M phase with hydroxyurea or nocodazole, respectively. Cell lysates were immunoprecipitated with anti-polyclonal Plk1 antibody and immunoblotted for Cdc6 or Plk1. As shown in Fig. 2C, endogenous Plk1 associated with endogenous Cdc6 in nocodazole-treated cells.

The reciprocal interaction between Plk1 and Cdc6 *in vitro* and *in vivo* suggests that Cdc6 may be a substrate of Plk1. To address this possibility, *in vitro* Plk1 kinase assay was carried out with purified His-tagged Cdc6 protein. GST-tagged wild type Plk1 (WT) or kinase-defective mutant (KM) was purified from baculovirus and incubated with Casein as a positive control or purified His-Cdc6 (Fig. 3A). Plk1-WT, but not Plk1-KM, phosphorylated Casein and His-Cdc6, indicating that Cdc6 is a substrate of Plk1 *in vitro*. To investigate the phosphorylation site of Cdc6 by Plk1, GST-tagged serial deletion mutants of Cdc6 were used for Plk1 kinase assay. Sequences at N-terminus of Cdc6 (aa 1-100) were phosphorylated by Plk1 (Fig. 3B). Using site-directed mutants of full-length Cdc6, Plk1 assays showed that the point mutant of Cdc6 containing T37V was not detectably phosphorylated by Plk1 *in vitro* (Fig. 3C), indicating that Cdc6 is phosphorylated by Plk1



on threonine 37. To address whether Plk1 phosphorylates Cdc6 in cells, Flag-tagged Plk1-WT and Plk1-KM were expressed in HEK 293T cells and then immunoblot assay was performed. Consistent with *in vitro* kinase assay, the Cdc6 band shift occurred in cells expressing Plk1-WT, but not in cells expressing Plk1-KM (Fig. S3). The results indicate that Cdc6 is a mitotic binding partner and substrate of Plk1.

### **The nonphosphorylatable mutant of Cdc6 induces defects in chromosomal segregation.**

To understand the physiological role of Cdc6 phosphorylation by Plk1, HeLa cells were synchronized in mitosis and stained for Cdc6 and Plk1 (Fig. 4A). Plk1 was detected in the centrosome and kinetochore during prophase, attached to the microtubule and the spindle pole in metaphase, localized in the central spindle in anaphase, and seen in the mid-body in telophase (Fig. 4A). Cdc6 co-localized with Plk1 during metaphase and anaphase at the spindle pole and the central spindle. In telophase, Cdc6 translocated to the newly formed nuclei (Fig. 4A). Co-localization of Cdc6 and Plk1 to the central spindle was consistently observed with different antibodies (Fig. S4).

Next, the effect of T37V mutant of Cdc6 (Cdc6-TV) on mitosis was studied. Cells expressing GFP-tagged Cdc6-TV were stained with anti-GFP and anti- $\alpha$ -tubulin. Over 40% cells expressing Cdc6-TV were bi-nucleated or had incompletely separated nuclei compared to 7% of cells expressing Cdc6-WT (Fig. 4, B-C), indicating that the nonphosphorylatable Cdc6 mutant may affect chromosomal segregation and cytokinesis.

### **The phosphorylation of Cdc6 by Plk1 regulates the activity of separase through the association with Cdk1.**

Chromosomal segregation in mitosis is triggered by activation of separase (26). In

vertebrates Cdk1-dependent inhibition of separase is a securin-independent pathway for anaphase control (27, 28). The association of separase and Cdk1 leads to inactivation of protease activity (28). To investigate the effects of Cdc6 phosphorylation by Plk1 on Cdc6 interaction with cyclin B1/Cdk1, co-immunoprecipitation assays were done. GFP-tagged Cdc6 was immunoprecipitated from GFP-tagged Cdc6-WT or Cdc6-TV transfected cells, and immunoblotted for Cdk1. Cdc6-WT but not Cdc6-TV interacted with Cdk1 (Fig. 5A). We also examined the association between Cdc6 and Cdk1 in Flag-tagged Plk1-WT or Plk1-KM transfected cells. Endogenous Cdc6 showed a different phosphorylation pattern after expression of Plk1-WT compared to Plk1-KM (Fig. 5B; Input). Immunoprecipitation assay showed that Cdc6 interacted with Cdk1 in Plk1-WT, but not in Plk1-KM expressing cells (Fig. 5B), suggesting that phosphorylation of Cdc6 by Plk1 induces the interaction of Cdc6 with Cdk1.

To determine whether the association of Cdc6 with Cdk1 affects Cdk1 activity, Histone H1 kinase assays were performed on Cdk1 immunoprecipitates. Expression of wild type Cdc6 significantly lowered the activity of Cdk1 compared to expression of the nonphosphorylatable mutant of Cdc6 or the GFP control (Fig. 5C), indicating that the phosphorylation of Cdc6 leads to inhibition of Cdk1 activity.

To determine whether the activity of separase is affected by the association of Cdc6 and Cdk1, the cleavage of Cohesin/Rad 21 was assayed. The cleavage of Cohesin/Rad21 was much greater in Cdc6-WT expressing cells than in GFP and Cdc6-TV mutant expressing cells. Expression of wild type Cdc6 but not mutant Cdc6 was correlated with activation of separase (Fig. 5D), indicating that the phosphorylation of Cdc6 by Plk1 and subsequent binding of Cdk1 leads to activation of separase and initiation of anaphase.

### **Cdc6 depletion induces mitotic defects and multinucleated cells.**

To test whether Cdc6 influences chromosomal segregation, Cdc6 was depleted with a Cdc6 targeting lentivirus. Twenty-four hours after infection, cells were selected with puromycin for two days, fixed and stained with anti-phospho-histone H3 (S10), a mitotic marker, and anti-Plk1. The level of phospho-histone H3 increased twofold in Cdc6-depleted cells compared to control cells (Fig. S5, A-B). Depletion of Cdc6 also increased the level of binucleated cells to 27 % compared to 4% in control cells (Fig. S5, A and C). FACS analysis performed four days after infection showed that the population of polyploid cells was 11.3% in Cdc6-depleted cells compared to 5% in control cells, and the population of subgenomic DNA was 23% in Cdc6-depleted cells compared to 3.7% in control cells (Fig. S5D). Depletion of Cdc6 also led to an increase the protein levels of Cdk1 and cyclin B1 (Fig. S5E). Cells failing to complete chromosomal segregation and cytokinesis accounted for 60% of the population of Cdc6-depleted cells (Fig. S5F and S6B). This phenotype was reduced to around 35% in Cdc6-WT-rescued cells or 15% in Cdc6-TV-expressed cells compared to control cells depleting Cdc6 (Fig. S6). Taken together, the results indicate that Cdc6 may have a critical role in chromosomal segregation and cytokinesis by regulating separase activity.

### **DISCUSSION**

Cdc6 is an essential regulator of DNA replication and plays a key role in origin licensing (10, 11, 13). The role of Cdc6 in mitosis is less well studied. Here we show that in mitotic cells Plk1 interacts with and phosphorylates Cdc6, and Plk1 and Cdc6 co-localize to the spindle pole and the central spindle. Phosphorylation of Cdc6 by Plk1 affects binding between Cdc6 and Cdk1 as well as Cdk1 activity. The interaction of Cdc6 with Cdk1

results in inhibition of Cdk1 activity, which is required for activation of separase and exit from mitosis. Mutation of Cdc6 results in cells exhibiting a high percentage of bi-nucleation and incompletely separated chromosomes. The depletion of Cdc6 also induces mitotic defects in chromosomal segregation and cytokinesis, and bi-nucleated cells with high levels of cyclin B1 and Cdk1.

Cdc6 is phosphorylated after initiation of DNA replication, and most of these sites depend on cyclin-dependent kinases (15, 21, 22). The phosphorylation of Cdc6 by cyclin A-Cdk2 is thought to play a role in translocation of Cdc6 from the nucleus to the cytoplasm to prevent DNA re-replication (21, 22, 23). However, after S phase is initiated an elevated Cdc6 is still found in the nucleus and is hyper-phosphorylated on serine 54 (29) before the completion of mitosis. Results presented here showing that endogenous Cdc6 associates with Plk1 and is localized with Plk1 at the spindle pole in metaphase and at the central spindle in anaphase (See Fig. 4A) suggest a role of Cdc6 in mitosis.

The phosphorylation of Cdc6 on threonine 37 may be critical for mitotic exit by regulating the interaction of Cdc6 and cyclin B1 or Cdk1. Cdc6 interacts with and inhibits mitotic CDK *in vitro* and *in vivo* through its amino terminus (30-33). Over-expression of a dominant-negative mutant of Cdc6 refractory to SCF<sup>CDC4</sup>-mediated proteolysis induces a mitotic delay correlated with inhibition of mitotic CDK activity (15, 30). This mutant inhibits CDK activity more efficiently than wild type Cdc6 because of its abundance (15, 32). Recently it has been reported that mitotic Cdc6 stabilizes APC/C substrates by a mechanism that is partially independent of mitotic CDK (17). Cdc6 downregulates APC/Cdc20 before cells exit mitosis through physical interaction with Cdc55, a subunit of PP2A phosphatase (17). Although it has been shown that overexpressed Cdc6 delays mitotic entry and progression in both a CDK-dependent and -independent manner, modest

levels of Cdc6 also acts to promote mitotic exit through inhibition of Cdk1 (33, 34). Removal of the Cdc6 N-terminus confers cytokinetic defects in *S. cerevisiae* deficient in Sic1 and Cdh1, suggesting that Cdc6, Sic1 and Cdh1 can cooperate to regulate mitotic CDK activity to facilitate mitotic exit (33, 34). Our studies also show that the nonphosphorylatable N-terminal mutant results in incompletely separated chromosomes, inducing failure of mitotic exit (Fig. 4 and S6). Moreover, cells co-expressing Cdc6-T37V along with Cdc25C, which promotes mitotic entry (35), still have a high percentage of mis-segregated chromosomes compared to cells expressing wild type Cdc6 and Cdc25C (unpublished data), suggesting that the phosphorylation of Cdc6 on threonine 37 is important for mitotic exit. In addition, Cdc6 depletion experiments reveal that endogenous Cdc6 is required to complete chromosomal segregation and cytokinesis in mitosis.

Activation and inactivation of Cdk1 are correlated with mitotic entry and exit (36). Cdk1 is highly activated at the beginning of mitosis and during mitotic progress (36). To pass through anaphase and exit mitosis, Cdk1 must be inactivated, which occurs when cyclin B is degraded (27, 36). In budding yeast, the Cdk inhibitors Sic1 and Cdc6 may contribute to down-regulation of Cdk1 activity in mitosis (31, 32). The N-terminus of Cdc6 is necessary and sufficient for interaction Cdk1 (16, 31) and phosphorylation of Cdc6 increases Cdk1 binding (31). Before anaphase, separase is inhibited by association with securin or Cdk1 (27, 28). The degradation of cyclin B1 and securin releases separase triggering chromosomal segregation (26, 28). In Cdc6-depleted cells, the levels of cyclin B or Cdk1 remain elevated, which may suppress the activation of separase. Chromosomal segregation was incomplete and cells were bi-nucleated or displayed incompletely separated chromosome. In addition, the cells expressing the Cdc6-TV mutant showed a similar phenotype. The Cdc6-TV mutant cannot associate with Cdk1 and cannot inhibit

Cdk1 activity. These data lead us to propose the following model (See Fig. 5E): in mitosis Plk1 phosphorylates Cdc6 which binds to Cdk1 and inhibits its activity. This leads to the release and activation of separase in a securin-independent manner, to promote chromosomal segregation.

## **MATERIALS AND METHODS**

### **Plasmid construction**

Full-length human Cdc6 was amplified by PCR and subcloned into pET-28a (His-fusion), pGEX-4T-1 (GST-fusion) and pCS2+GFP (GFP-fusion) vector (37, 38). Flag-tagged Plk1-WT, K82M, FAA, N-terminus, and C-terminus and baculovirus encoding wild type GST-Plk1 and GST-Plk1-K82M were described previously (24). The T37V mutant of Cdc6 was generated by replacing ACA with GTA (forward AGTGATGCCAAACTAGAACCCAGTAAATGTCCAAACCGTAACCTG; reverse CAGGTTACGGTTTGGACATTTACTGGTTCTAGTTTGGCATCACT).

### **Lentivirus-based RNAi plasmid preparation, virus production, and infection**

The lentivirus-based RNAi transfer plasmids targeting human Cdc6 (gene access number NM\_001254) at positions 1314 to 1333 (GGACAATGCTGCAGTTCAATT) (pLKO-Puro1.-Cdc6) or control plasmid (pLKO-Puro.1) was prepared and lentivirus was generated and infected as described previously (19, 20).

### **Phosphatase treatment**

HeLa cells were synchronized with the double thymidine block. Nine hours after release, cells were harvested and lysed in phosphatase buffer (150 mM NaCl, 1% CHAPS, 10mM HEPES, pH 7.5) and either left untreated or incubated for 30 minutes at 37 °C with alkaline

phosphatase (BioLabs, USA). At the end of the reaction the lysates were immunoblotted with specific antibodies as described in SI Materials and Methods.

### **GST pull down assay**

Glutathione S-transferase (GST) fused Cdc6 expressed in *E. coli* strain BL21 and purified using Glutathione-Sepharose 4B beads were incubated with the lysates of HEK 293T cells that had been expressed with flag-tagged Plk1 as described in SI Materials and Methods.

### **Kinase assay**

For Cdk assays, cyclin A or cyclin B was immunoprecipitated from lysates from cells treated with hydroxyurea for 12 h or nocodazole for 12 h, respectively. Activity was assayed with histone H1 as detailed in SI Materials and Methods.

Plk1 kinase activity was assayed with purified His-tagged full length Cdc6, GST-tagged regions of Cdc6, or Casein as described in SI Materials and Methods.

### **Immunofluorescence**

For immunofluorescence, cells were fixed, permeabilized, incubated in 0.1% Triton X-100-PBS containing 3% BSA for blocking, incubated with anti-monoclonal Plk1 (Upstate) and anti-Cdc6 (ProteinTech Group) antibodies, and analyzed as described in SI Materials and Methods.

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## FIGURE LEGENDS

**Figure 1. The phosphorylation of Cdc6 does not occur in Plk1-depleted cells, and is independent of the activities of cyclin-dependent kinases. (A)** Cell extracts at 9 h after

release from the double thymidine block were incubated with or without 50 U alkaline phosphatase (BioLabs) for 30 min at 37°C and immunoblotted with anti-Cdc6, anti-Plk1, anti-Cdt1, anti-Mcm7 and anti- $\beta$ -tubulin antibodies. **(B)** Cells were infected with lentivirus-expressing RNAi targeting Plk1 (Plk1-) or control virus (Con). Twenty-four hours later, cells were selected with puromycin for 2 days and then lysates were immunoblotted with anti-Plk1, anti-Cdc6, and anti-Erk2 antibodies. **(C)** HeLa cells were synchronized with the double thymidine block and infected with lentivirus-based RNAi targeting Plk1 (Plk1-) or with control virus (Con). Zero time is 24 h after infection. Lysates were prepared at various times and immunoblotted. FACS analysis of cell cycle progression was performed. **(D –G)** HeLa cells were infected with lentivirus-expressing RNAi targeting Plk1 (P) or control virus (C). Twenty-four hours later, cells were selected with puromycin for 36 hours and then treated with 2 mM hydroxyurea or 100 ng/ml nocodazole for 12 hours. **(D and F)** Cdk activity was assayed with immunoprecipitated cyclin A- or cyclin B1-dependent kinase with anti-cyclin A or anti-cyclin B1 antibody and histone H1 was used as substrate. **(E and G)** Extracts were immunoblotted with anti-cyclin A, anti-cyclin B1, anti-Cdk1, anti-Cdk2, anti-Plk1, anti-Cdc6, and anti-Erk2 antibodies.

**Figure 2. Plk1 interacts with Cdc6 through its C-terminal polo-box.** **(A)** GST-tagged Cdc6 purified from *E.coli* and bound Sepharase beads were incubated with lysates of HEK293T cells transfected with pCMV-Flag or pCMV-Flag-tagged Plk1. The Flag-tagged Plk1 associated with GST-tagged Cdc6 was detected by immunoblot analysis with anti-Flag antibody. GST-tagged Cdc6 was detected by anti-GST antibody. **(B)** Flag, Flag-tagged wild type Plk1 (WT), Polo-box mutant (W414F, V415A, L427A; FAA), N-terminal (N), and C-terminal (C) Plk1 were expressed in HEK293T cells for 2 days and then extracts were immunoblotted with anti-Flag (Input). Purified GST-Cdc6 was incubated with lysates of

these cells and the Flag-Plk1 protein associated with GST-Cdc6 was detected by immunoblot with anti-Flag antibody. GST-Cdc6 was detected with anti-GST antibody. (C) HeLa cells were treated with 2 mM hydroxyurea or 100 ng/ml nocodazole for 12 hours. Extracts were immunoprecipitated with anti-monoclonal Immunoglobulin G or anti-monoclonal Plk1 and then immunoblotted with anti-Cdc6, Plk1 or Erk2 antibodies. One-fourth of total input is also shown. The supernatant is one-fifth of total proteins.

**Figure 3. Plk1 phosphorylates Cdc6 on residue T37.** (A) Purified GST-tagged wild type Plk1 (WT) or kinase-defective mutant (KM) was assayed with Casein or His-tagged Cdc6 as a substrate and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P] ATP at 30°C for 30 min. (B) GST tagged Plk1-WT assayed with purified GST-tagged regions of Cdc6, amino acids 1-100 (100), 101-204 (204), 205-410 (410), 411-560 (560) as substrates and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P] ATP. (C) GST tagged Plk1-WT was assayed with purified His-tagged full-length wild type Cdc6 (WT) or T37V mutant Cdc6 (T37V) and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P] ATP. The samples were resolved with SDS-PAGE and detected by autoradiography.

**Figure 4. The nonphosphorylatable mutant of Cdc6 induces defects in chromosomal segregation.** (A) HeLa cells grown on coverslips were synchronized with the double thymidine block. Nine hours after release, the cells were fixed and stained with anti-monoclonal Plk1 (Upstate; green) and anti-polyclonal Cdc6 (PreteinTech; Red). Nuclear DNA was stained by DAPI. The scale bar indicates in 10  $\mu$ m. (B-C) GFP, GFP-tagged wild type Cdc6 (Cdc6-WT), T37V mutant Cdc6 (Cdc6-TV) were expressed in HeLa cells for 2 days. Cells were fixed and stained with anti-GFP and anti- $\alpha$ -tubulin. The scale bar indicates in 20  $\mu$ m. The populations of bi-nucleated cells were counted and quantified. The means  $\pm$  SD (error bars) from three determinations obtained in each of three experiments.

**Figure 5. Phosphorylation of Cdc6 by Plk1 regulates separase activity via interaction**

**with Cdk1.** (A) GFP-tagged mock (GFP), wild type Cdc6 (WT), and T37V mutant of Cdc6 (TV) were expressed in HeLa cells for 2 days. Extracts were immunoprecipitated with anti-GFP, and then immunoblotted with anti-Cdk1 antibody. Input was analyzed by western blot with anti-GFP, anti-Cdk1, and anti-cyclin B1 antibodies (B) Flag-tagged mock, Plk1-WT, and Plk1-KM proteins were expressed in HeLa cells for 2 days and then the cells were treated with nocodazole for 12 h. Extracts were immunoprecipitated with anti-Cdc6 and then immunoblotted with anti-Cdk1. Input was analyzed by western blot with anti-Cdc6, anti-Flag, anti-Cdk1, and anti-Erk2 antibodies (C-D) Cells were prepared as described in Figure 5A. (C) Extracts were immunoprecipitated with anti-Cdk1 and Cdk1 activity was assayed with Histone H1 and 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P] ATP at 30°C for 30 min. Activity was detected by autoradiography. (D) Extracts were immunoblotted with anti-Cdk1, anti-Rad21, anti-GFP, anti-GAPDH antibodies. Three independent experiments were done. (E) Plk1-mediated phosphorylation of Cdc6 leads the activation of separase. Cdc6 phosphorylated by Plk1 binds with Cdk1 and inhibits its activity. Thus, separase may be released from Cdk1, which promotes chromosomal segregation in mitosis.

Figure 1

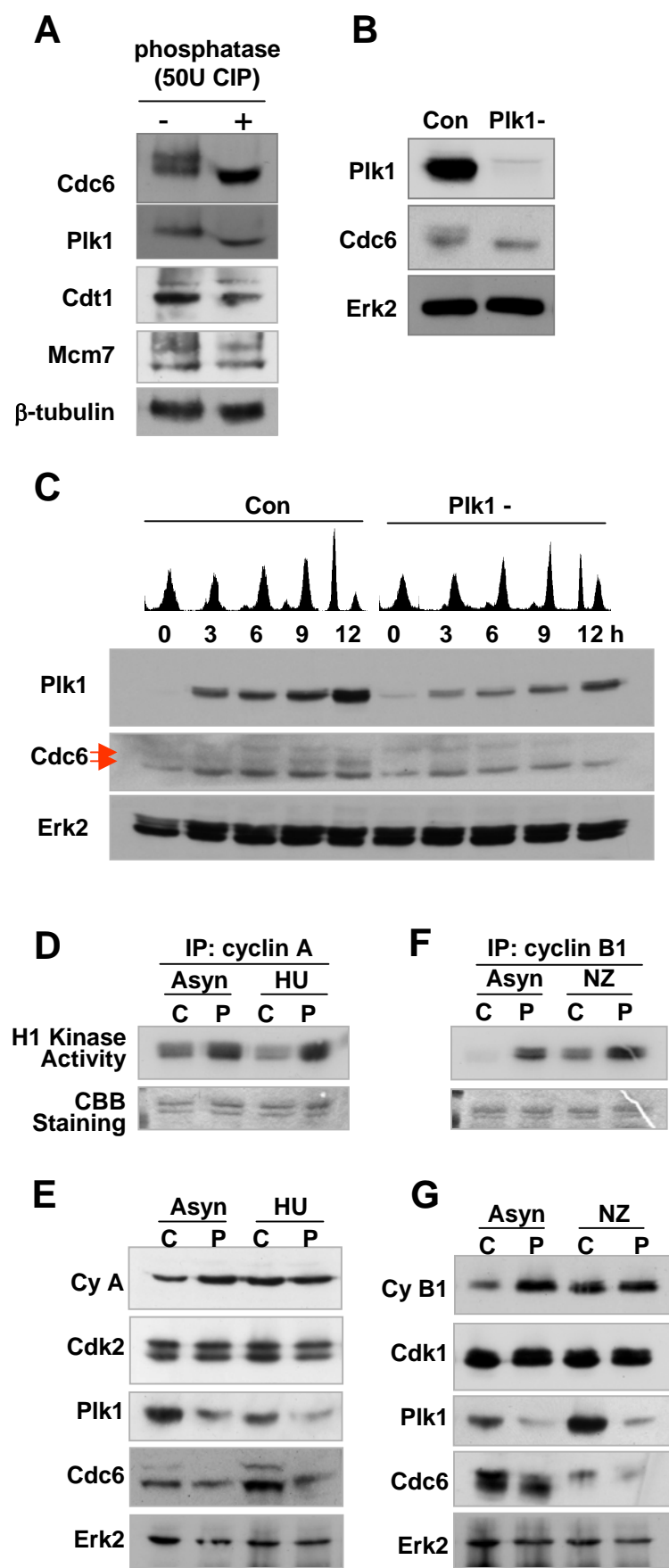


Figure 2

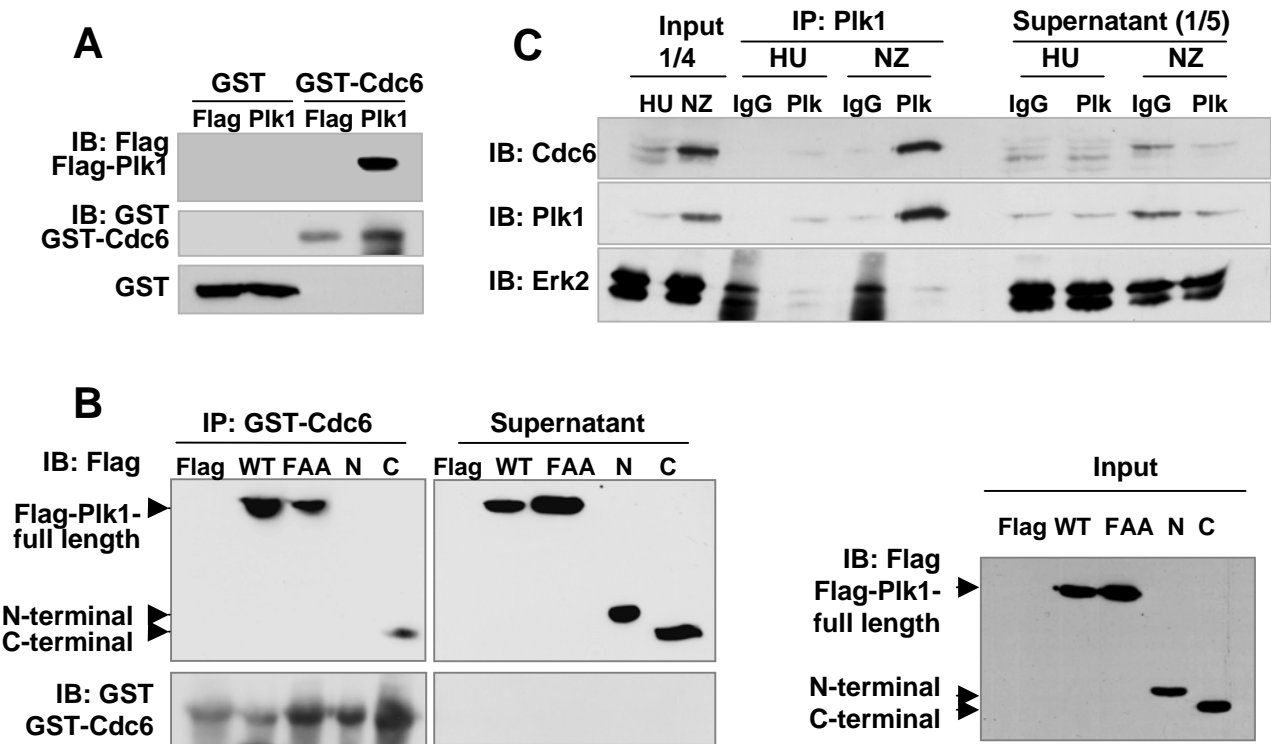
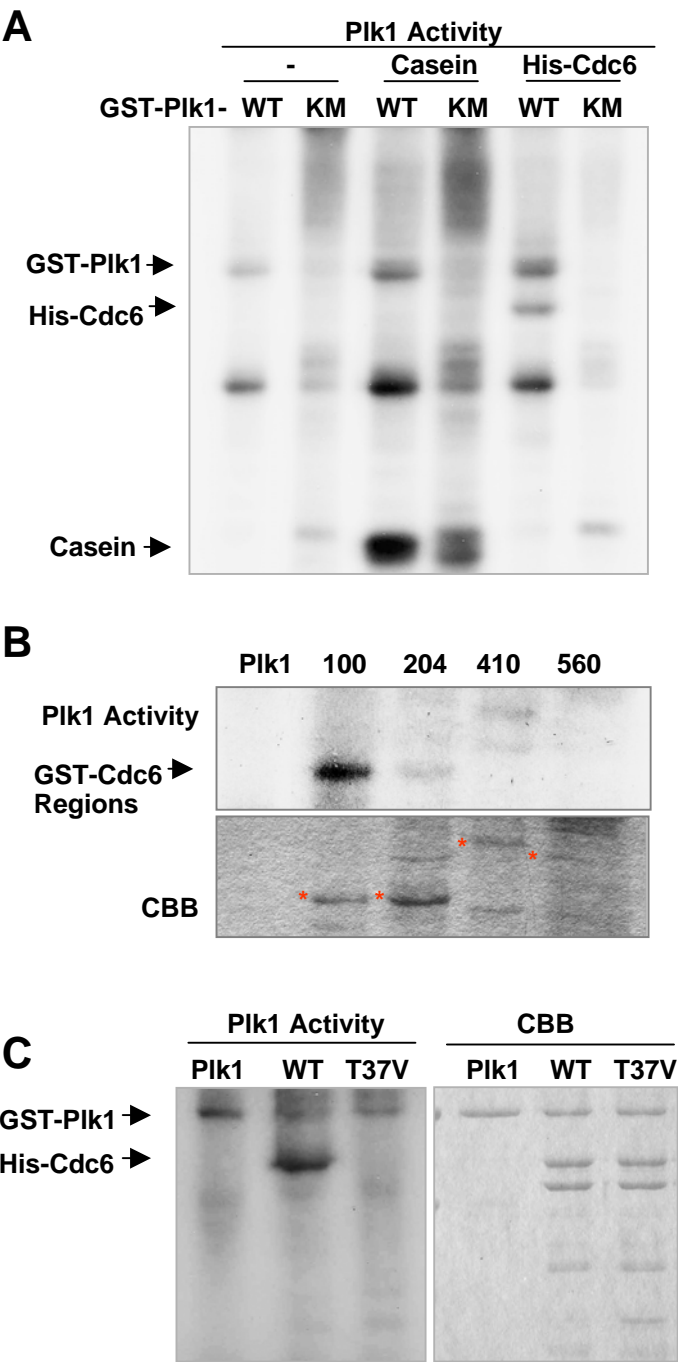
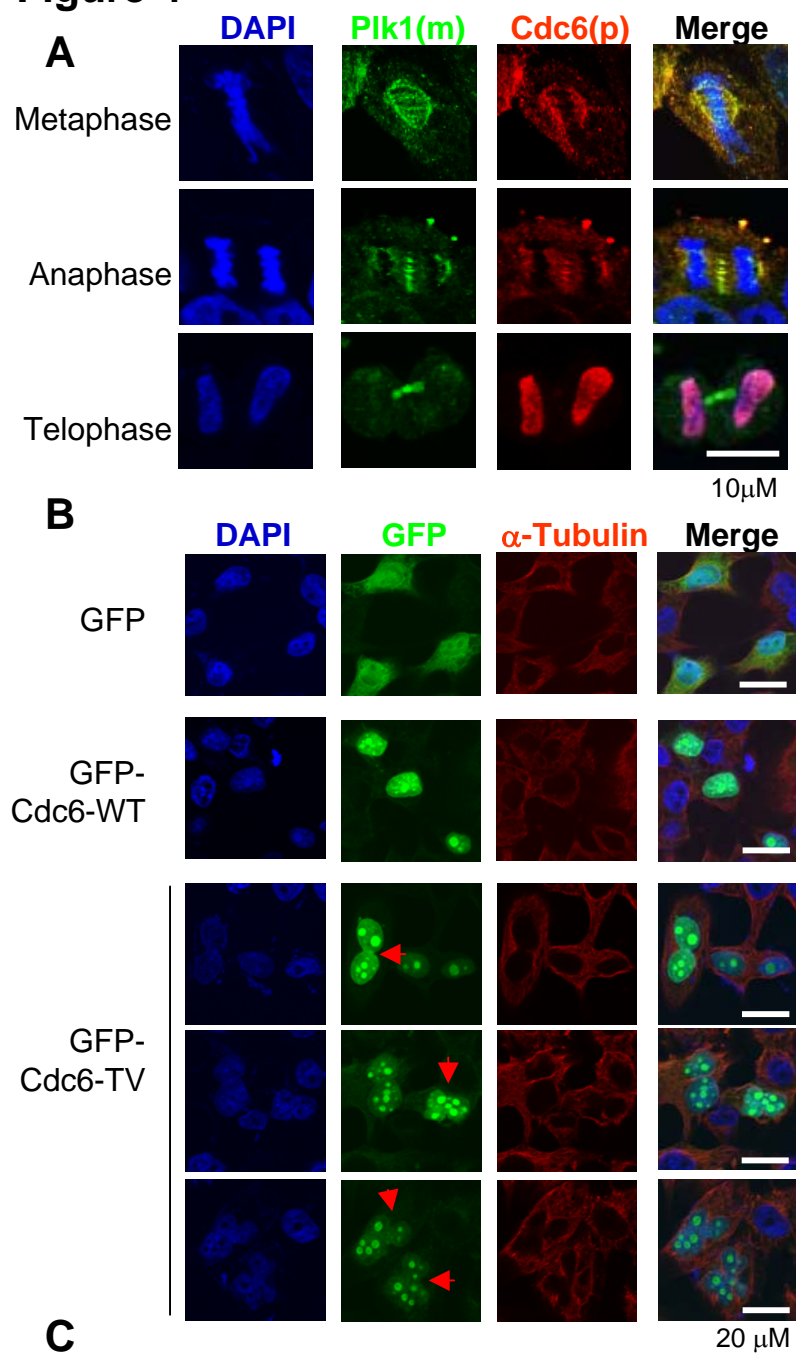




Figure 3



**Figure 4**



**Figure 5**

